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Absence of humic substance reduction by the acidophilic Fe(III)-reducing strain *Acidiphilium* SJH: implications for its Fe(III) reduction mechanism and for the stimulation of natural organohalogen formation

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Abstract A vast amount of volatile organohalogens (VOX) has natural origins. Both soils and sediments have been shown to release VOX, which are most likely produced via redox reactions between Fe(III) and quinones in the presence of halide anions, particularly at acidic pH. We tested whether acidophilic Fe(III)-reducers might indirectly stimulate natural VOX formation at acidic pH by providing reactive Fe and quinone species. However, it is unknown whether acidophilic Fe(III)-reducers can reduce humic acids (HA) or fulvic acids (FA). We therefore tested the ability of the acidophilic Fe(III)-reducer *Acidiphilium* SJH to reduce macromolecular, suspended HA and dissolved FA at pH 3.1–3.3. We found that (i) SJH can neither reduce HA/FA nor the humic model quinone anthraquinone-2,6-disulfonic-acid (AQDS) nor stimulate the formation of FA radicals, (ii) at acidic pH, significantly more electrons are transferred abiotically both from native and reduced FA to dissolved Fe(III) than from native or reduced HA, and (iii) the presence of strain SJH does not stimulate VOX formation. Our results imply that the acidophilic Fe(III)-reducer SJH either uses an enzyme for Fe(III) reduction that can neither be used for HA/FA nor for AQDS reduction or that the location of Fe(III) reduction is inaccessible for these compounds. We further conclude that microorganisms such as strain SJH probably do not indirectly stimulate natural VOX formation at acidic pH via the formation of reactive quinone species.

Keywords Acidophilic Fe(III)-reducers · Humic and fulvic acid reduction · Natural organohalogen formation

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Introduction

Volatile halogenated organic compounds (VOX) play an important role in atmospheric chemical processes including stratospheric ozone destruction (Molina and Rowland 1974) and global warming (WMO 2010). Some of these compounds have mainly natural sources, e.g. methyl chloride (Butler 2000). Several recent studies suggest that a considerable amount of CH_3Cl might originate from decaying plant material in tropical soils (Keppler et al. 2005; Saito et al. 2008; WMO 2010). However, details of reaction pathways, the exact role soils play in the global cycling of VOX as well as the parameters controlling emissions from this source remain to be elucidated. In batch experiments and soil incubations performed at pH 2–3, VOX yields increased tremendously with the

addition of H_2O_2 (Keppler et al. 2002; 2006; Huber et al. 2009), suggesting a Fenton-like reaction and an important role of hydroxyl- or semiquinone radicals. Since the presence of an active microbial community has been shown to be crucial for VOX emission from sediments (Weissflog et al. 2005), we hypothesized that microbial radical formation via humic substance reduction could stimulate VOX formation and therefore intended to evaluate the influence of microorganisms on VOX emissions from soil and sediment.

Humic substances (HS) are polymeric, heterogeneous redox-active organic compounds formed during the degradation and transformation of biopolymers such as lignin, proteins and carbohydrates. HS consist of three operationally defined fractions: humins, which are non-soluble at both acidic and alkaline pH; humic acids (HA), which are alkaline-soluble and acid-insoluble (with a small portion being soluble at neutral pH); and fulvic acids (FA), which are soluble at both acidic and alkaline pH (Stevenson 1994). The group of microorganisms that can reduce dissolved humic substances at circumneutral pH includes Fe(III)-reducers (Lovley et al. 1996), fermenting bacteria (Benz et al. 1998), toluene degraders, sulfate-reducers and methanogens (Cervantes et al. 2002). It has been shown recently that even solid-phase humic substances can serve as electron acceptors for bacteria (Roden et al. 2010).

All neutrophilic Fe(III)-reducing bacteria so far tested have been able to transfer electrons to humic substances or to the humic model quinone AQDS (anthraquinone-2,6-disulfonate) (Lovley et al. 1996; Coates et al. 1998; Francis et al. 2000; Nevin and Lovley 2000; Finneran et al. 2002; He and Sanford 2003). During this process, organic radicals are formed (Scott et al. 1998). In order to analyze if microorganisms could indirectly stimulate natural organohalogen formation in low-pH soil environments by providing organic radicals, we tested whether one specific acidophilic Fe(III)-reducer, *Acidiphilium* strain SJH, can reduce HS and produce semiquinone radicals similar to neutrophilic Fe(III)-reducers.

For neutrophilic Fe(III)-reducers it has been demonstrated in a recent study that under pH neutral conditions, the same *c*-type cytochromes distributed over both cell membranes and the periplasm are involved in electron transfer to both humic substances and Fe(III) oxides (Voordeckers et al. 2010). However, the reduction of chelated, and therefore dissolved Fe(III), which can enter the periplasm e.g. via ligand-gated outer membrane-receptors (Andrews et al. 2003), does not necessarily depend on the cytochrome electron transport chain (Leang et al. 2005; Mehta et al. 2005). This strongly suggests that an additional Fe(III)-reduction mechanism must exist within neutrophiles that mediates electron transfer to dissolved Fe(III) which might be located in the periplasm and has been suggested to be used for AQDS reduction as well (Lies et al. 2005). At this point, the question arises whether acidophilic Fe(III)-reducers can also reduce macromolecular HA and FA, or at least the quinone model compound AQDS, as it is small enough to enter the periplasm via porins.

Recent research has shown that the potential for dissimilatory ferric iron reduction is widespread among acidophilic heterotrophic bacteria (Coupland and Johnson 2008). This makes sense considering the fact that Fe(III) in the acidic solutions represents a favorable electron acceptor for energy generation by microorganisms at low pH. An explanation for this can be found in the standard redox potential of the ferrous/ferric iron couple being +770 mV at pH 2 in comparison to −112 mV at neutral pH (assuming 1 mM dissolved Fe(II)) (Stumm and Morgan 1996). In our study we used an acidophilic bacterium with particularly high rates of Fe(III) reduction, *Acidiphilium* strain SJH. SJH is a heterotrophic, facultative anaerobic α-proteobacterium which has been isolated from acid mine fluids (Johnson and McGinness 1991). This strain can reductively dissolve a variety of Fe(III) minerals such as goethite and akageneite (Bridge and Johnson 2000) and couple Fe(III) reduction to growth under both oxic and anoxic conditions (Johnson and Bridge 2002). The mechanism of Fe(III) reduction in SJH is only poorly understood. This bacterium was suggested to excrete a heat-stable compound (e.g. a small organic ligand but not an enzyme) that enhances the dissolution of Fe(III)-containing minerals, but does not catalyze Fe(III) reduction itself (Bridge and Johnson 2000). The localization of the Fe(III)-reducing enzyme(s) in this acidophile remains subject of further research.

In order to elucidate whether microbial radical formation via humic substance reduction at acidic pH (by acidophilic Fe(III)-reducers) could stimulate

VOX formation, in the present study we intended to determine (i) whether non-dissolved HA can serve as electron acceptors for the acidophilic Fe(III)-reducer *Acidiphilium* SJH. Further we wanted to test, (ii) whether this strain can reduce fulvic acids or the model quinone AQDS, both of which are dissolved under acidic conditions, and (iii) whether VOX are formed in cultures of SJH in presence of Fe(III), HA/FA and chloride. These results will then allow to draw conclusions regarding the potential indirect microbial influence of acidophilic Fe(III)-reducing microorganisms such as strain SJH on natural VOX formation.

Materials and methods

If not stated otherwise, sterile and anoxic conditions were maintained for preparation of all solutions as well as during sampling. All experiments were set up in an anoxic chamber (glovebox) under N_2 atmosphere and incubated at 28°C in the dark. Details concerning composition and incubation conditions of the experimental setups are given in Table 1.

Bacterial cultures and media

Acidiphilium strain SJH was kindly provided by DB Johnson (Bangor University) and cultivated in liquid medium containing 15 mM $(NH_4)_2SO_4$, 1.3 mM KCl, 3 mM K_2HPO_4 and 2 mM $MgSO_4 \times 7\ H_2O$ (medium 269 of the German Collection of Microorganisms and Cell Cultures, DSMZ). The pH was adjusted to pH 3.0 with 1 M H_2SO_4 and if the medium was used for cultivation, glucose and yeast extract were added after autoclaving to final concentrations of 0.1% and 0.03% (w/v), respectively. Cultures were kept at 28°C under oxic conditions and shaken at 200 rpm. For reduction experiments, the same mineral medium was prepared in a Widdel flask and cooled to room temperature under an N_2 stream. If necessary, pH was readjusted to pH 3.0 with 1 M H_2SO_4, before the medium was dispensed into 50 ml glass bottles without addition of glucose or yeast extract. 10 mM glycerol was added from a 500 mM stock solution. Experiments were set up with 5-day old cultivation cultures that were harvested at the end of their logarithmic growth phase. In the beginning of an experiment, cells were quantified by optical density (OD) measured at 600 nm according to a calibration curve plotting OD_{600} against microscopic cell counts with a Neubauer chamber (cell number per ml $= 6 \times 10^8 \times OD_{600} + 10^7$). 1.2 times as many cells as needed (to account for the loss during centrifugation/washing) were centrifuged for 10 min at 9,000*g* and washed with mineral medium twice. After the third centrifugation step, cell pellets were resuspended in mineral medium to a concentration that required 100 or 250 µl of the resulting cell suspension to be added per setup in order to give rise to final cell densities of 10^7 or 5×10^8 cells per ml, respectively. Final cell densities were verified by microscopic counts of DAPI-stained cells for selective setups at the beginning and end of batch experiments.

Preparation of native and chemically reduced humic and fulvic acid solutions

Hohloh Lake fulvic acids were kindly provided by Christian Zwiener (University of Tübingen). A detailed characterization of these fulvic acids is published in (Gul et al. 2003). Pahokee Peat humic acids (PPHA) were purchased from the International Humic Substance Society (IHSS). HA suspensions and FA solutions were prepared in mineral medium 269.

Final concentrations of 5 or 10 mg/ml of the respective HA and FA were prepared in medium under constant stirring. During this process, all FA dissolved while the majority of HA remained in suspension. The pH was adjusted to pH 3.0 with NaOH until it remained stable for at least 10 min. FA solutions and HA suspensions were transferred into 100 ml glass bottles in batches of 60 ml and closed with butyl rubber stoppers and metal crimp caps. Native (non-reduced) HA suspensions and FA solutions were deoxygenated by applying 3 cycles of 3 min vacuum followed by 1 min flushing with N_2. For chemical reduction of FA solutions and HA suspensions, 5 palladium-coated aluminum pellets (0.5% Pd, Merck) were added per bottle prior to degassing by application of vacuum for 30 min. FA solutions and HA suspensions were then flushed with H_2 for 2 min and put on a rotary shaker for 16 h. Prior to the use of FA solutions and HA suspensions in further experiments, headspaces were exchanged with N_2 as described above in order to avoid Fe(III) reduction by residual H_2 in the Fe(III)-NTA assay (Roden et al. 2010). Both native and chemically

Table 1 Overview about composition and incubation conditions of five separate experimental setups and the respective control experiments (two different control experiments per experimental setup)

Experiment	Composition and number of setups	Composition and number of controls	Incubation conditions
HA[a] reduction experiment	Medium 269	Medium 269, 5 mg/ml chemically reduced PPHA	Anoxic, 5 h
	5 mg/ml native PPHA[b]	10 mM glycerol (3 setups)	
	10 mM glycerol	Medium 269, 5 mg/ml native PPHA	
	5×10^8 cells per ml	10 mM glycerol (3 setups)	
	(3 setups)		
FA reduction experiment	Medium 269	Medium 269, 5 mg/ml chemically reduced PPHA	Anoxic, 5 h
	5 mg/ml native FA[c]	10 mM glycerol (3 setups)	
	10 mM glycerol	Medium 269, 5 mg/ml native PPHA	
	5×10^8 cells per ml	10 mM glycerol (3 setups)	
	(3 setups)		
AQDS reduction experiment	Medium 269	50 mM phosphate buffer pH = 4.7	Anoxic, 4 weeks
	2 mM AQDS[d]	2 mM chemically reduced AQDS	Oxic, 4 weeks
	10 mM glycerol	10 mM glycerol (1 setup)	
	10^7 cells per ml	Medium 269, 2 mM AQDS	
	(6 setups)	10 mM glycerol (2 setups)	
VOX formation experiment	Medium 269	Medium 269, 5 mg/ml chemically reduced PPHA	Anoxic, 3 weeks
	5 mg/ml native PPHA	10 mM glycerol	
	10 mM glycerol	35 mM dissolved Fe(III) (3 setups)	
	35 mM dissolved Fe(III)	Medium 269, 5 mg/ml native PPHA	
	5×10^8 cells per ml	10 mM glycerol	
	(3 setups)	35 mM dissolved Fe(III) (3 setups)	
ESR experiment	Medium 269	Medium 269, 10 mg/ml chemically reduced PPHA	Anoxic, 5 h
	10 mg/ml native FA	10 mM glycerol (3 setups)	
	10 mM glycerol	Medium 269, 10 mg/ml native PPHA	
	5×10^8 cells per ml	10 mM glycerol (3 setups)	
	(3 setups)		

[a] Humic Acids

[b] Pahokee Peat Humic Acids

[c] Fulvic Acids

[d] Anthraquinone-2,6-Disulfonic Acid

reduced FA solutions were additionally filtered through 0.22 μm cellulose ester filters into evacuated and sterilized bottles. HA suspensions were not filtered in order to leave the precipitated HA particles in the reaction mixture. For determination of the redox state of chemically reduced HA (see below), HA samples were taken directly from the suspensions after brief shaking by hand followed by rapid sedimentation of the Pd-Al-pellets to the bottom of the vial thus leaving the palladium-coated alumina pellets behind.

Setup of microbial HA and FA reduction experiments

The experiments to determine if SJH can reduce HA and FA (HA and FA reduction experiments) were set up in 20 ml glass vials with 10 ml total volume. Chemically reduced HA suspensions and FA solutions (for preparation see previous section) served as positive controls for the microbial reduction experiments. First, glycerol was added to the HA- or FA-containing medium followed by addition of the

cells. Both incubation as well as sampling at $t = 0$ and $t = 5$ h and the Fe(III)-NTA assay took place within the anoxic chamber. The content of dissolved organic carbon (DOC) was quantified, after filtration and appropriate dilution of samples, by a total organic carbon (TOC) analyzer (Elementar, Hanau, Germany).

Determination of redox state of humic and fulvic acids by the Fe(III)-NTA assay

The redox state of HA suspensions and FA solutions before and after 5 h incubation with SJH as well as before and after chemical reduction was determined with Fe(III)-nitrilotriacetic acid (Fe(III)-NTA) as described by (Roden et al. 2010) with the only modification that 0.5 ml of sample was incubated with 1 ml of 5 mM Fe(III)-NTA.

Quantification of VOX formation in Fe-HA experiments

Experiments to quantify volatile organohalogens (VOX) formed in microbially stimulated versus abiotic setups were prepared in the same way as the HA reduction experiments. However, here 35 mM dissolved Fe(III) was added from a 500 mM $Fe(III)_2(SO_4)_3$ stock solution to every setup and the glass vials were closed with PTFE-layered butyl rubber septa to enable gas chromatography-mass spectrometry (GC-MS)-measurements of volatile organohalogens. Organohalogens were then quantified by GC-MS as described by (Huber et al. 2010). Details about instrumentation, temperature program and detection limits are given in online resource 5.

AQDS reduction experiments

The experiments to determine if SJH can reduce AQDS were set up in a similar way to the FA and HA reduction experiments, i.e. in 20 ml glass vials with 10 ml total volume. 3 parallel setups were prepared anoxically and closed using butyl rubber stoppers. 3 parallel setups were covered by loose aluminum caps enabling exchange with atmospheric O_2 during incubation. AQDS reduction to the hydroquinone form was followed visually by a color change from translucent (AQDS) to yellow (AH_2QDS). Chemically reduced AH_2QDS in the same medium was prepared as a positive control by reduction via the Pd/H_2-treatment described above. Since the pH increased from pH 3 to pH 7 due to the addition of Pd pellets (even without/before H_2 addition), chemical reduction of AQDS with Pd/H_2 was also performed in 50 mM phosphate buffer that was adjusted initially to pH 3. However, even in the 50 mM phosphate buffer, the pH increased after addition of Pd pellets from pH 3 to pH = 4.7.

Electron spin resonance (ESR) spectroscopy

For quantification of organic radicals by ESR spectroscopy, 3 parallel setups containing 10 mg/ml FA were adjusted inside an anoxic chamber to pH 12.0 with NaOH to stabilize the organic radicals that had formed before and after 5 h incubation with strain SJH. Samples from chemically pre-reduced and native FA solutions in absence of SJH were prepared for comparison. Samples were added to glass capillaries (Blaubrand Intramark Mikropipettes, Brand GmbH, Germany) and sealed with a vinyl sealing kit (Haematocrit Sealing Compound, Brand GmbH, Germany). They were then placed into quartz tubes with an inner diameter of 4 mm and closed with plastic caps (Magnettech GmbH, Berlin, Germany). To limit penetration of O_2 into the samples throughout transport and measurement, the tubes were additionally sealed with parafilm. Quartz tubes were then analyzed at the Federal Institute for Materials Research and Testing (BAM) in Berlin where ESR spectra were recorded at 25°C using an ESR spectrometer (MiniScope MS 300, Magnettech GmbH, Berlin, Germany) at a modulation amplitude of 2000 mG, a damping of 20 dB and an amplification of 900 with 3 runs of 30 s per spectrum.

Results and discussion

In order to evaluate whether acidophilic microorganisms might stimulate natural organohalogen formation by producing organic radicals, we tested the ability of *Acidiphilium* strain SJH to reduce HA, FA and AQDS in comparison to its ability to reduce Fe(III).

Fe(III) reduction by strain SJH under both oxic and anoxic conditions

In order to first evaluate the Fe(III) reduction capacity of SJH, we monitored Fe(III) reduction over time both under anoxic and microoxic conditions. Since we were interested in maximum rates of Fe(III) reduction, we used Fe(III) sulfate as electron acceptor, which is almost completely soluble at pH 2–3 leading to high concentrations of dissolved Fe(III) at the pH at which our experiments were conducted. It has been shown before that SJH cannot use sulfate as an electron acceptor (Johnson and McGinness 1991). Figure S1A shows that an initial inoculum of 10^7 cells/ml reduced 4 mM Fe(III) to Fe(II), i.e. approx. 80% of the initially present 5 mM Fe(III), with 10 mM glycerol as an electron donor within 35 days.

In order to compare the extent of Fe(III) reduction by SJH under microoxic and anoxic conditions, we repeated the same experiment under microoxic conditions. Figure S1C shows that all Fe(III) got reduced under microoxic conditions within 27 days, while under anoxic conditions, only around 10% got reduced within the same time. This can be explained by the around 14-fold increase in cells numbers we observed in the microoxic, but not in the anoxic setups (Fig. S2).

To find out whether cell suspension experiments can be applied to test reduction by strain SJH, we first determined whether strain SJH can reduce Fe(III) in cell suspensions, in addition to the observed Fe(III) reduction in growing batch cultures. In these cell suspension experiments, we found that with 5×10^8 cells/ml, approximately 1 mM of dissolved Fe(III) was reduced within 5 h under anoxic conditions (Fig. S1B).

Absence of reduction of fulvic and humic acids and AQDS by strain SJH

As strain SJH reduced Fe(III) efficiently under anoxic conditions, we determined whether strain SJH could also reduce fulvic and/or humic acids under the same conditions. To this end, SJH cells were incubated with Pahokee Peat HA or Lake Hohloh FA as electron acceptor and glycerol as electron donor. Even though SJH had been shown to reduce Fe(III) at much higher rates under oxic than under anoxic conditions, anoxic conditions were chosen for these kinds of experiments in order to avoid reoxidation of FA, HA and AQDS by atmospheric oxygen. As positive controls, HA and FA were reduced chemically by Pd/H_2 and were incubated with glycerol under the same conditions as in the experimental setups, but without addition of cells. Since it had been previously demonstrated that the reducing capacities of HA after chemical reduction are very similar to the values obtained for microbial reduction (Peretyazhko 2006), we considered chemically reduced HA and FA as suitable positive controls to assess the capacity of strain SJH to reduce them microbially. At the beginning and at the end of incubation of strain SJH with the HA and FA, the reduction state of the humic or fulvic acids was determined by quantifying the amount of electrons that were transferrable from the HA and FA to Fe(III)-NTA (Fig. 1).

We found that native (non-reduced) HA reduced only low amounts of Fe(III) in the Fe(III)-NTA assay before and after incubation with glycerol, demonstrating that the glycerol did not chemically reduce the HA (Fig. 1). In contrast, chemically reduced HA reduced approximately 8 times more Fe(III) than the native HA. Incubation of the reduced HA with glycerol in the absence of cells did not lead to an increased reducing capacity again indicating that the glycerol did not reduce the HA chemically. We then tested whether addition of SJH cells changed the reducing capacity of the HA. However, incubation of native HA in the presence of SJH and glycerol showed no evidence for electron transfer from the cells to the HA. We therefore concluded that strain SJH is the first Fe(III)-reducer tested that is unable to reduce humic acids, although it can reduce Fe(III). Since we used relatively high concentrations of HA and FA, the absence of microbial HS reduction at these conditions suggests that microbial reduction of HS at acidic pH is not relevant at high and therefore probably also not at low HS concentrations. By using high concentrations of HA and FA, we increased the sensitivity of our assays and thus prevented overlooking low extents of reduction of HS as it could potentially be the case at lower HS concentrations.

Because the cells were present during the short 1 min Fe(III)-NTA assay, we performed control experiments with cells alone (no HA and no FA) to determine whether they influenced the Fe(III)-NTA assay and could show that no detectable microbial

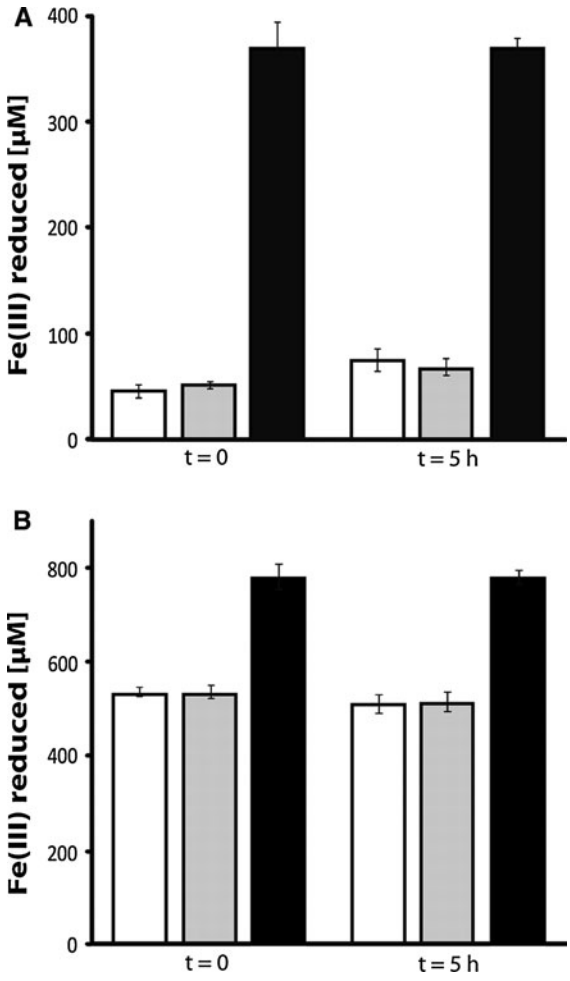

Fig. 1 Amount of Fe(II) formed by incubation of humic acids (**a**) and fulvic acids (**b**) with Fe(III)-NTA before (0 h) and after incubation (5 h) with (*grey bars*) and without (*white bars*) the acidophilic Fe(III)-reducing strain SJH and glycerol as electron donor. *Black bars* represent humic or fulvic acid solutions which had been first chemically reduced by H_2/Pd for 18 h and then incubated for 0 or 5 h with glycerol (no microbial cells present). Experiments were performed with **a** Pahokee Peat humic acid and **b** Lake Hohloh fulvic acid. Please note the different maximum values at the y-axes. Standard deviation/error bars were calculated from results of triplicate setups

Fe(III) reduction by cells from biotic setups takes place within the 1 min reaction time (data not shown).

Potential reasons for the absence of humic substance reduction by strain SJH

In order to understand the absence of HA reduction by strain SJH the solubility and molecular size of the HA have to be considered. Even though the pH of our medium and HA, as well as FA stock solutions, had been adjusted to 3.0, the different compositions and reactions within the individual setups caused slight pH changes over the incubation periods. Therefore, we measured a final pH of 1.8 in the Fe(III) reduction setups, a pH of 3.1 in the FA and AQDS reduction setups and a pH of 3.3 in the HA reduction setups. The pH values of the positive controls where the FA and HA solutions had been reduced by Pd-H_2 were slightly (0.2 to 0.4 pH units) higher than the pH values of the experimental setups after incubation. However, these slightly higher pH values were also measured in control setups with HA and FA solutions which had been incubated with Pd under N_2 atmosphere (in absence of H_2) for 16 h. Samples from these control setups did not reduce any additional Fe(III) in the Fe(III)-NTA assay compared to samples from setups prepared with native FA/HA solutions that had never encountered any Pd and had a slightly lower pH (Fig. S4). Therefore, we can rule out any effect of small pH deviations or residual traces of Pd in some setups on the Fe(III)-NTA assay including Fe quantification.

Within the pH range of our experiments, FA molecules are soluble, in contrast to HA for which only a very small part is dissolved. DOC measurements of 0.22 µm-filtered setups without glycerol showed that approximately 4.5 mg/ml FA but only 0.5-0.7 mg/ml HA were in solution in the individual setups and that most of the HA were present in non-dissolved state. Since also under neutral pH conditions most humic substances are non-dissolved but can still be used by Fe(III)-reducing microorganisms as electron acceptor (Kappler et al. 2004; Roden et al. 2010), our data suggest that not all Fe(III)-reducing microorganisms can reduce dissolved and non-dissolved humics. This implies that different Fe(III) reduction pathways are present in known neutrophilic Fe(III)-reducers than in the acidophilic Fe(III)-reducing strain analyzed in this study.

Since it had been suggested that the poorly described second Fe(III) reduction pathway for dissolved, and thus easily accessible, Fe(III) that occurs within neutrophilic Fe(III)-reducers might be involved in reduction of soluble quinones (Voordeckers et al. 2010), we hypothesized that strain SJH can reduce AQDS and FA. While the FA are dissolved but probably too large to enter the periplasm to a large extent, AQDS is both dissolved and small

enough to enter the periplasm (Shyu et al. 2002). However, our experiments showed that strain SJH can neither reduce FA (Fig. 1b) nor the model quinone compound AQDS (no color change to yellow could be observed neither upon incubation under oxic or anoxic conditions while chemically reduced AH_2QDS turned clearly yellow even at acidic pH (Fig. S4). This suggests that even if the enzyme that reduces Fe(III) in acidophiles is located in the periplasm, it is not only unable to reduce quinones due to a lack of accessibility (in the case that they are too large to enter the periplasm) but also due to a lack of specificity for quinone moieties.

Interestingly, in the experiments with FA we observed a much higher background Fe(III) reduction in the Fe(III)-NTA assay by the native FA when compared to the HA experiments. More than 10 times more Fe(II) was formed by native FA (values of 531 μM) than by native HA (values in the range of 46 μM), independent of whether cells were present or whether the samples had been taken before or after incubation with cells (Fig. 1).

Chemically reduced FA also produced twice as much Fe(II) as chemically reduced HA. This data first suggests a general higher reducing capacity of the FA compared to the HA used in our experiments, and second, points towards a relatively reduced redox state of the native FA.

This is in agreement with previous experiments showing that native FA can reduce several times more Fe(III) at acidic pH than native HA (Szilágyi 1971; Skogerboe and Wilson 1981). These authors explain this finding by citing the higher redox potential of HA suspensions than of FA solutions at pH 2. The reduction potential of a HA suspension at pH 2 versus the normal hydrogen electrode has been determined to be +700 mV (Szilágyi 1973), while for FA a value of +500 mV has been reported (Skogerboe and Wilson 1981). These values are slightly below the redox potential of the Fe^{3+}/Fe^{2+}-couple of +770 mV at pH 2 (Stumm and Morgan 1996) but definitely positive enough to render their reduction with glycerol as an electron donor thermodynamically favorable.

Abiotic processes between Fe(III)-NTA, glycerol and HA/FA

In order to determine to which extent abiotic reactions, in particular glycerol-HA and glycerol-FA interactions might have influenced the Fe(III)-NTA assay, we set up batch experiments where dissolved Fe(III) was incubated with either FA or HA in the presence and absence of glycerol. For comparison, dissolved Fe(III) was incubated with glycerol alone. We found that glycerol alone did not reduce any Fe(III) (Fig. 2). However, within a couple of days, we quantified 6 mM Fe(II) in the HA-containing setups and even 15 mM Fe(II) in the FA-containing Fe(III)-NTA setups both in the presence and absence of glycerol (a) confirming the reduction of Fe(III) by native HA and FA as described above and (b) suggesting that the glycerol did not further increase or influence this reaction, i.e. HA and FA are not reduced by the glycerol.

Absence of radical formation during incubation of humic substances with strain SJH

In addition to the quantification of electron transfer from humic substances to Fe(III), we used radical measurements by ESR spectroscopy to investigate microbial reduction of FA under acidic pH conditions. In contrast to microbial reduction experiments with FA and HA at neutral pH where high radical concentrations were found (Jiang et al. 2009), in our setups where solutions of 10 mg/ml FA were incubated with strain SJH at acidic pH, no radical formation could be detected by ESR measurements (data not shown). Moreover, incubation of FA with H_2/Pd at pH 2.3 also did not lead to increasing concentrations of radicals, although increasing

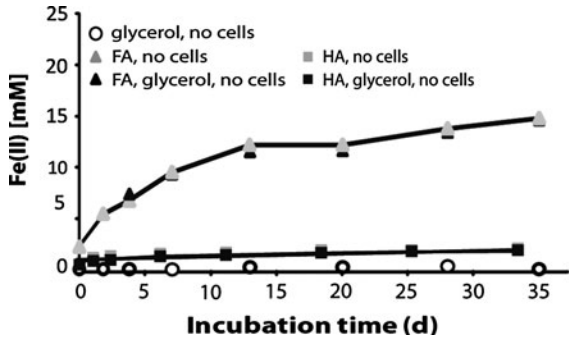

Fig. 2 Fe(II) formation over time during abiotic incubation of 40 mM Fe(III) with 10 mM glycerol (white circles), 5 mg/ml FA (grey triangles), 5 mg/ml HA (grey squares), 10 mM glycerol plus FA (black triangles), or 10 mM glycerol plus HA (black squares). Setups were prepared sterilely without addition of cells and incubated for 35 days under anoxic conditions

reducing capacities were observed (Fig. 1). This suggests that radical measurements are not suited to follow reduction of HA and FA under acidic conditions since under these conditions reduction obviously leads—in contrast to reduction at neutral pH—exclusively to the hydroquinone state without significant accumulation of semiquinone radicals. This is in line with results from a modeling study by (Rosso et al. 2004) who calculated that at acidic pH, a two-electron transfer to AQDS to the hydroquinone AH_2QDS is thermodynamically much more favorable than a one-electron transfer to form semiquinone. Additionally, (Ratasuk and Nanny 2007) showed that below pH 6.5, most likely non-quinone functional groups serve as electron acceptors in humic substances, which additionally explains why we did not observe any semiquinone radical formation at pH 2.3.

Microbial influence on natural organohalogen formation

In order to test our initial hypothesis, namely whether VOX are formed in cultures of Fe(III)-reducers in presence of Fe(III), HA and chloride, we performed GC-MS measurements of setups with native PPHA in presence and absence of SJH and with chemically reduced PPHA without bacteria. With regard to our target compounds, namely volatile organohalogens that have been shown to be produced naturally in previous studies, chloromethane (CH_3Cl) and bromomethane (CH_3Br) were detected in some setups in the low nanogram range (data not shown). However, calculated in parts per trillion volume (pptv), the CH_3Cl concentrations we measured were about twofold lower than environmental background concentration of about 600 pptv as given by WMO (2010). For CH_3Br, the concentrations we measured exceeded the environmental background concentration of 7.5 pptv (WMO 2010) about 200-fold. However, similar amounts of these compounds were detected in all setups including both abiotic control setups and microbially active setups. This means that bromomethane might have formed in our setups but its formation was not enhanced by the presence of the acidophilic Fe(III)-reducing strain *Acidiphilium* SJH. Since SJH can neither reduce HA/FA or AQDS at acidic pH nor cause semiquinone formation of these compounds at pH 3, this result could be expected. Consequently, we propose that this acidophilic Fe(III)-reducer cannot enhance natural organohalogen formation in acidic environments by providing organic radicals. However, (Huber et al. 2009) showed that the Fe(III)- and radical-dependent oxidative way of VOX formation strongly decreases with increasing pH, such as at pH > 3.6, and hardly any trihalomethane formation could be observed any more. Therefore, based on our results, we hypothesize that microorganisms do not stimulate the radical-dependent mechanism of organohalogen formation via formation of reactive Fe and humics species. Instead we propose that in the pH-neutral salt lake sediments, where a clear dependency of VOX formation on the presence of an active microbial community has been shown (Weissflog et al. 2005), microbes rather mediate the organohalogen formation in a direct way, e.g. via haloperoxidase-like enzymes, which have already been shown to be present in soil (Asplund et al. 1993).

Implications for mechanisms of electron transfer to Fe(III) in neutrophilic and acidophilic Fe(III)-reducing microorganisms

The present study also revealed details regarding the potential electron transport pathway(s) to Fe(III) and the selectivity of the responsible Fe(III)-reducing enzymes in the acidophilic Fe(III)-reducing microorganism *Acidiphilum* strain SJH in comparison to neutrophilic Fe(III)-reducers (Fig. 3). Neutrophilic Fe(III)-reducers have to cope with a poorly soluble electron acceptor, i.e. Fe(III) minerals. To overcome this problem, three strategies for electron transfer are known to exist within neutrophilic Fe(III)-reducers. These include direct contact between outer membrane Fe(III)-reductases and the Fe(III) mineral and electron transfer mediated by outer membrane cytochromes (DiChristina et al. 2002; Clarke et al. 2011). Additional strategies are the use of microbially produced or external electron shuttles (e.g. dissolved or solid-phase humic substances (Jiang and Kappler 2008; Roden et al. 2010) as well as the excretion of organic ligands that solubilize Fe(III) which can then be taken up by the bacteria and reduced by a Fe(III) reductase located in the periplasm or in the inner membrane (Pitts et al. 2003; Fennessey et al. 2010). Reduction of Fe(III) and humic substances by outer membrane reductases that depend on electron flow over several quinone- and *c*-type cytochrome-containing proteins in the inner

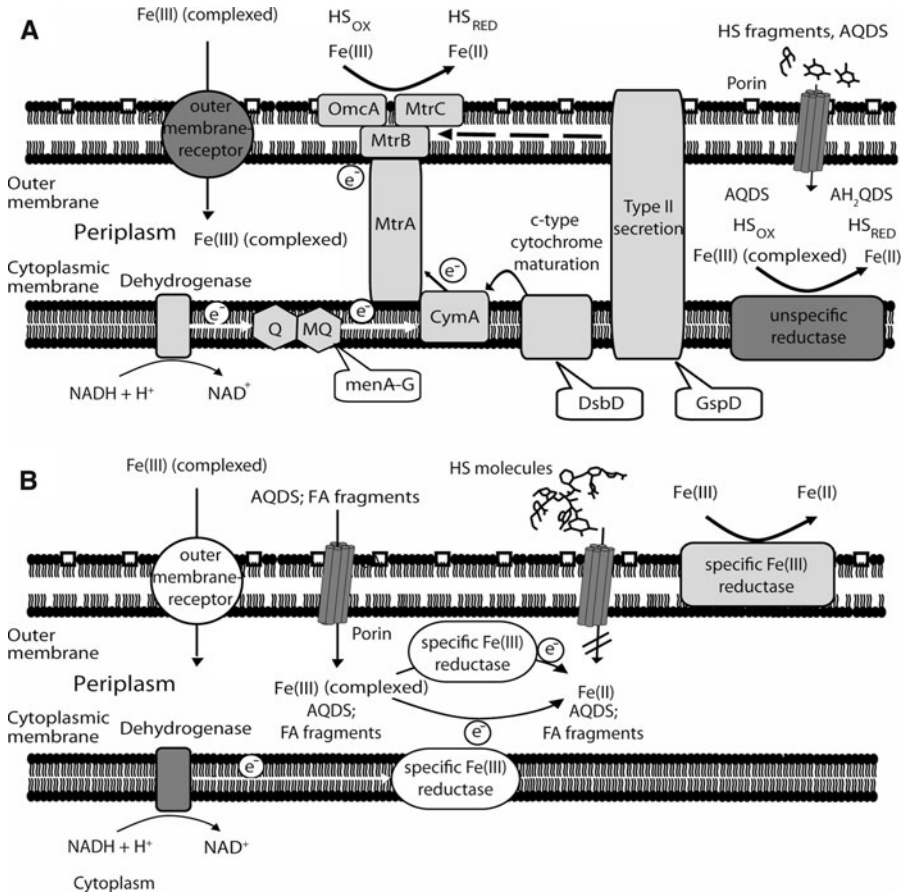

Fig. 3 Mechanisms of microbial Fe(III) and humic substance reduction **a** by neutrophilic Fe(III)-reducers and **b** by the acidophilic Fe(III)-reducer *Acidiphilium* strain SJH as proposed in this study. Previous studies indicate that at least 2 ways for Fe(III) and humic substance reduction exist in neutrophiles including (1) cytochrome-dependent electron transfer leading to a terminal reductase at the outer membrane (involved proteins are depicted in light grey) and (2) reduction of membrane-permeable quinones and dissolved (complexed) Fe(III) in the periplasmic space or at the inner membrane (involved proteins are depicted in dark grey). Reduction of complexed Fe(III) by a periplasmic- or inner-membrane reductase is also a possible mechanism in acidophiles (hypothetically mediated by proteins depicted in white in panel **b**). Alternatively, this enzyme could also be located at the outer membrane (depicted in light grey). Based on our results, the Fe(III) reducing enzyme of SJH does not accept any quinones as electron acceptor, as it does in the case of neutrophiles

membrane and the periplasm have recently been shown to be based on the same molecular machinery (Lies et al. 2005; Voordeckers et al. 2010). Electron transfer to dissolved Fe(III) by periplasmic electron transfer components has been suggested to represent an additional pathway of Fe(III) reduction for neutrophiles and to mediate reduction of dissolved and outer-membrane-permeable quinones such as AQDS (Lies et al. 2005). It is very well feasible that at neutral pH even FA and possibly also HA fragments can be reduced by this mechanism. This means that no Fe(III) reductase is known to exist within neutrophiles that could not transfer electrons either to HA/FA or to dissolved quinones.

The lacking ability to reduce HA/FA and AQDS by the Fe(III) reductase of strain SJH could point either to a difference in localization or specificity of this Fe(III) reductase in comparison to Fe(III) reductases of neutrophiles. The main difference between Fe(III) reduction at acidic versus neutral pH is the up to 10^{18} fold higher availability of dissolved iron (Fe^{3+}) in acidic as compared to neutral environments which goes along with an astoundingly large number and diversity of Fe(III)-uptake systems of acidophilic

microorganisms (Osorio et al. 2008). This suggests that Fe(III) reduction in acidophiles could occur in the periplasm or even in the cytoplasm. However, since acidophiles maintain a circumneutral pH in the cytoplasm as neutrophiles do (Hsung and Haug 1977; Oshima et al. 1977; Cox et al. 1979), an uptake of the Fe(III) into the cytoplasm would lead to a loss of the thermodynamic advantage to reduce dissolved (free) Fe^{3+} at low pH (since the Eh of redox couples of Fe^{2+}/complexed Fe(III) are less positive than the Eh of Fe^{2+}/Fe^{3+}). This renders a cytoplasmic location of the Fe(III) reductase in acidophilic Fe(III)-reducing microorganisms highly unlikely, even though we cannot completely exclude this possibility. The fact that filtered heat-killed cells and SJH spent medium could mediate dissolution, but not reduction of Fe(III)-containing minerals (Bridge and Johnson 2000) suggests that the Fe(III) reductase of this strain is not a secreted enzyme either. This means that if the lacking ability of SJH to reduce HA/FA and AQDS is attributable to a localization of the Fe(III) reductase that renders it accessible for dissolved and/or complexed Fe(III), but not for HA/FA and AQDS, it would make sense to expect the enzyme to be located in the periplasm (or at the periplasmic side of the inner membrane). The most common way for small molecules to enter the cytoplasm is via porins which allow the passage of particles with a size up to 600 Dalton (Da) into the periplasm (Nikaido 1992). This means that AQDS, which is below this size, should be able to enter the periplasm. The size of fulvic acids varies between 500 and 2000 Da (Stevenson 1994), implying that at least a fraction of them should be able to pass the porins. Humic acids are generally believed to consist of larger molecules than fulvic acids with a size of up to 250 kDa (Stevenson 1994) and therefore it would not be expected that they can enter the periplasm to a significant extent. It has to be mentioned, however, that recent studies have suggested that HA are rather large aggregates of relatively low molecular size molecules than large polymers (Sutton and Sposito 2005). This means that small fragments could be released from these aggregates and could potentially be taken up into the periplasm. Based on the conclusion that AQDS, a part of the FA and potentially even some HA molecules probably enter the periplasm but still did not become reduced, we infer that if the Fe(III)-reducing enzyme of acidophiles such as SJH is located at the outer membrane or in the periplasm, it obviously differs from Fe(III) reductases of neutrophiles in terms of a narrower specificity for complexed Fe(III) excluding quinone compounds.

In summary, this suggests that the Fe(III) reduction pathways of neutrophilic Fe(III)-reducers can also transfer electrons to quinones such as AQDS and HA/FA, but this is not the case for the Fe(III) reduction mechanism of the acidophilic strain used in this study. Further studies are needed to elucidate whether this finding can be generalized, and if the mechanism of Fe(III) reduction in acidophiles is fundamentally different from the mechanisms of Fe(III) reduction that have been described for neutrophiles.

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